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13. ABSTRACT (Maximum 200 Words)

Most research that requires long-term propagation of prostate cancer (PCA) cells is carried out with three cell lines: DU 145, PC-3, and LNCaP. All but one of these lines, LNCaP, fail to express prostate specific antigen (PSA), androgen receptors, and/or any other prostate phenotype. Some prostatic fluids contain prostate cancer cells. Our goals were (1) to test the tumorigenicity of and to develop transplantable xenografts from PCA cells in prostatic fluid, (2) to develop methods for enhancing the tumorigenicity of small numbers of these PCA cells without deliberately altering their genes, (3) to test these methods for enhancement of tumorigenicity with prostatic fluid cells, and (4) to initiate clinical follow-up. As detailed in the body of our report, our aims that required prostatic fluid have been limited by exceptionally weak clinical collaboration that has become progressively weaker. The fluids that we tested earlier were promising. Samples in the past year have been quite inadequate. Coinjection of lethally irradiated, growth-factor-producing cells was encouraging in some experiments in the case of some tumors; however, results were quite variable in repeated experiments. The inadequacies in fluid samples and the repeatability of experiments are detailed in the body of this report.

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ARMY ANNUAL REPORT (YEAR 3)

Introduction

The development of new approaches to the treatment of prostate cancer (PCA) would be greatly facilitated if there were a larger number of experimental models available. As we reviewed (Pretlow and Pretlow, 2000a) in a discussion of models of prostate cancer previously, (a) the rodent prostate seems almost irrelevant to us since it is biochemically, functionally, and embryologically quite different from the human prostate and (b) there are very limited numbers of human PCA epithelial cells that can be grown in the laboratory. Tissue culture lines derived from human PCAs are successful in less than 0.1% of human PCAs tested, and most such lines fail to make two molecules that are very important for most human PCAs: androgen receptor and prostate specific antigen (PSA). There are laboratories, including our own, that have reported the development of PCA xenografts from 5-10% of human primary PCAs; and most such xenografts make both PSA and androgen receptors, i.e., have functional properties in common with most human PCAs. If an investigator wants to develop a human PCA tissue culture line from a xenograft, there is an infinite supply of xenograft available, and tissue culture lines have been established from xenografts. One such line was developed from one of our xenografts (Sramkoski et al., 1999) and is available from the American Type Culture Collection (their catalogue number CRL-2505). Our goals in the proposed research have been to assess the tumorigenicity of PCA cells in prostatic fluid, to develop methods to enhance the tumorigenicity of the small number of cells available there, to test those methods for the enhancement of tumorigenicity of small numbers of PCA cells, and to initiate clinical follow-up to find any correlations that may exist between the clinical courses of patients and whether or not their cells grow to form xenografts.

The research carried out during the first three years of this project leads to two conclusions. Firstly, it is feasible to maintain cells that make PSA, probably prostate cancer cells, from the prostatic fluids of many patients with prostate cancer after subcutaneous transplantation in nude mice for many months. It is likely that a proportion of these patients' fluids would lead to serially transplantable prostate cancer xenografts. We did not receive a sufficient number of fluids of high quality to test this hypothesis definitively. A sine qua non for success in this kind of research is a collaborating urologist who makes the effort to massage the prostate consistently and appropriately. Secondly, the coinjection assay that we developed for the evaluation of irradiated "feeder" cells that make growth factors is imprecise. This assay might be useful in the hands of a pharmaceutical company with a sufficient budget to carry out large numbers of expensive experiments; however, the lack of precision in this assay would mandate a very large number of experiments if one were to achieve the unequivocal proof of the growth-promoting effects of coinjected, irradiated feeder cells. This same lack of precision raises serious questions about the value of coinjected "feeder" cells in testing cells from different patients for tumorigenicity.

Body

Specific Aim 1 (also task 1). To test the tumorigenicity of cells obtained from the prostatic fluids (PF) of patients with PCA and to develop serially transplantable xenografts from these cells.

In months 1-18, we (statement of work, task 1 of the proposal) proposed to test the tumorigenicity of prostatic fluid cells obtained by prostatic massage from 30 patients. Our testing was delayed initially because our institution renovated some of the nude mouse facility and did not finish that task until approximately four months after this grant started. This restricted severely the number of cages available to our laboratory for several months. As detailed below, we obtained sufficient cells for injection from eighteen patients during the first two years of this project. In the third year, we received sufficient cells for injection from no patients. We injected cells from the prostatic fluids of patients into nude mice, when

sufficient cells were found on cell count. Blood has been drawn from these mice 1, 2, 4, and 6 months (as the mice reached those intervals) after the injection of the cells. Elevated blood PSA has been observed in mice injected with prostatic fluid cells from several patients; however, the elevated PSA has been of low magnitude. To date, we have not observed tumor histologically at the site of injection; however, we did observe tumor in the lungs of one mouse injected with PF cells (see below).

Because this aspect of our work is probably complete, I shall list the results that we obtained from work with the samples obtained. Each sample was divided between two mice. The numbers in the column at the left of the table represent prostatic fluid samples from individual patients. As an example, prostatic fluid from patient number one in the table was divided equally between mouse #9680 and mouse #9682. Mouse #9680 had one occasion when his blood PSA was abnormal at 0.10 ng/ml. This occurred four months after the injection of prostatic fluid. Some animals had PSA elevated on more than one occasion; for example, animal # 11626 had detectably elevated PSA at the intervals 1 and 4 months after injection of prostatic fluid. Animals 10028 and 10030 are presented in more detail below.

	mouse	PSA values (ng/ml) >0.02	Time post-injection
1	9680	0.10	4 months
	9682	0	
2	9728	0.04	1 months
	9730	0.03	2 months
3	9764	0	
	9766	0	
4	9882	0	
	9884	0.18	2 months
5	9886	0	
	9888	0	
6	10028	0.03-1.67*	see below
	10030	0.03-2.13*	see below
7	10060	0	
	10062	0.04	4 months
8	10068	0	
	10070	0.04	7
9	10160	0.04	2
	10162	0	
10	10172	0	
	10174	0	
11	10256	0.06-0.09	6-12 months
	10258	0.08-0.17	2-10 months
12	10924	0.03-0.06	2-6 months
	10926	0.04-0.09	1-6 mOnths

	10972	0.04	1 month
14	11170	0.04	1 month
	11172	0.03	1 month
15	11472	0.14	4 month
	11474	0.13	4 month
16	11594	0	
	11596	0.03	4
17	11626	0.03-0.41	1-4 months
	11628	0	
18	11630	0.03	2
	11632	0.20	4

Time post -injection	1mo	2mo	4mo	5mo	6mo	8mo	10mo	12 mo	12.5 mo	13 mo*
mouse										
10028	0.03	0.02	0.14	0.62	0.92	0.90	1.67	1.24	1.20	1.06
10030	0.02	0.02	0.03	0.22	0.50	1.15	2.13	1.45	1.14	1.11

*at time when killed

Both animals 10028 and 10030 received cells from the same sample of prostatic fluid and showed very similar patterns of PSA in their blood. I would speculate that the 0.03 ng/ml value seen in animal 10028 at one month resulted from cells many of which did not live as long as two months, since values in both animals were normal at two months. Both animals showed values that increased regularly starting at four months and declined regularly after reaching maxima at ten months. Both animals were killed thirteen months after transplantation of the prostatic fluid cells.

Animal # 10030 was killed a week before 10028, since 10030 had the highest PSA and we hoped to find tumor to transplant. Because we found no tumor at autopsy, we speculated that our best chance of recovering transplantable tumor cells would be in the injection site. It was excised, digested serially with Pronase as used by us previously for xenografts (Wainstein et al., 1994) and described by us previously in detail for use with human prostatic tissues (Helms et al., 1975). The resultant cell suspension was divided so that each of two mice (#s 11672 and 11674) received four million cells in Matrigel subcutaneously. One of these animals developed a PSA of 0.06 ng/ml at two months after injection; however, this level declined to 0.05 ng/ml at four months and undetectable after six and eight months. The other recipient mouse never developed detectable PSA in his blood. Neither of these animals showed evidence of tumor at autopsy.

Animal #10028 showed a tumor that measured 3x3x1 mm in maximum dimensions in his left lung. That tumor and the injection site were dissociated with Pronase (as above) and injected into two animals each subcutaneously in Matrigel. None of these four animals developed detectable PSAs in their blood.

I would conclude that (a) PSA-producing cells (probably prostate cancer cells) from the prostatic fluid of patients with prostatic carcinoma do survive in nude mice for many months in some cases. An effective approach to stimulating them to grow more rapidly would be a prerequisite to having them form serially transplantable xenografts.

MAJOR PROBLEM IN CLINICAL COLLABORATION: Our first attempt to evaluate prostatic fluid was stimulated by our reading of several papers by Bologna that we reviewed

previously (Pretlow, T. G. and T. P. Pretlow, 2000a). In our initial evaluation of prostatic fluid, we wanted to know if Bologna was simply euphoric or if a large proportion of prostatic fluids from patients with prostate cancer really contain prostate cancer cells. Among human cells, only bone marrow stem cells and malignant cells grow in soft agar in culture. We obtained prostatic fluids from sixteen patients immediately prior to prostatectomy. The samples obtained from five of these sixteen patients contained too few cells to merit culture. Prostatic fluids from the remaining eleven patients contained an average of average of 6.13 million cells per sample. One of these was infected with bacteria. Of the samples from the remaining ten patients, samples from eight patients grew as "colonies" and/or "clusters" in soft agar, a behavior that shows that samples of prostatic fluid from eight of sixteen prostate cancer patients contained prostate cancer cells.

Following this set of patients, we had a series of approximately sixty patients whose prostatic fluids contained fewer than a million cells per sample. This was both frustrating and injurious to our laboratory, since we repeatedly prepared for cells in fluids that then contained few cells or no cells. After discussing this with Dr. Resnick (all of our samples came from Dr. Resnick), I learned nothing. I also discovered, by accident, that many of the prostatic fluid samples that we had been promised were being sent, without my knowledge, to an assistant professor in Dr. Resnick's department for another purpose. Neither Dr. Resnick nor that assistant professor had ever done laboratory experiments with prostatic fluid until my results were known to them.

I then called Dr. Resnick's long-term secretary and asked who the residents were when our initial sixteen patients were examined. She identified those residents as being Dr. Vafa and Dr. Wainstein. Both of these physicians had left our institution and were practicing urologic surgery privately. I called both of them and explained my problem. Without any hints from me and without the opportunity to contact each other, both of these urologists expressed their views that Dr. Resnick was probably not in the operating room and had probably not instructed the residents adequately with regard to obtaining adequate samples by massaging prostates preoperatively.

I then explained this problem to a Dr. Sommers who was Head of Urology in Akron, forty-two miles from my laboratory. We commuted to Akron for many samples in collaboration with Dr. Sommers. More than half of the samples that we obtained from Dr. Sommers contained adequate numbers of cells, and the results in culture were similar to those that we had observed with the first sixteen patients.

I then visited Dr. Resnick and informed him about (a) the opinions of Drs. Vafa and Wainstein and (b) our experience with Dr. Sommers. Dr. Resnick stated that he would try again. After that, we obtained a short series that resulted in my being sufficiently optimistic to consider writing this grant for the Army. I discussed the feasibility of writing this grant with Dr. Resnick. He estimated that we have at least a hundred patients with primary prostate cancer who would be eligible donors of prostatic fluid. Because of this assurance, I wrote a grant that would require thirty samples/year.

Consistent with my previous experience with Dr. Resnick, the collaboration has been inconsistent but generally very poor. The first four successive samples from patients immediately prior to prostatectomy contained sufficient cells. The next five patients failed to give fluid with sufficient cells. We decided to get fluid from patients immediately prior to brachytherapy. Eight out of eight patients failed to yield sufficient cells. I have several memoranda to Dr. Resnick in my files that describe my problem.

Specific Aim 2 (also task 2). To develop methods for enhancing the tumorigenicity of small numbers of PCA cells without deliberately altering their genes.

Specific Aim 2 is particularly important since the successful conduct of the proposed work for specific aim 3 is dependent on the identification of tumor cells that can be irradiated and

coinjected with CWR22 with enhancement of the rate of growth of CWR22. Success in specific aim 2 or any other successful approach to recruiting a larger proportion of human prostate cancer cells into the proliferative fraction might also have enormous significance for areas that are not directly related to this research proposal. For example, there are those who are interested in growing PCA cells from the blood of patients with PCA. We reported the growth as xenografts of PCA cells from the blood of two of eleven patients with metastatic PCA (Pretlow et al., 2000b); however, if this is to provide a useful means of sampling patients' metastatic tumor for the purpose of predicting the appropriate drugs for specific patients, any means that would permit the growth of PCA xenografts from a higher proportion of PCA patients would greatly facilitate this kind of research.

As detailed in previous annual reports, for these experiments marginally tumorigenic doses of CWR22 are coinjected with graded doses of lethally irradiated tumor cells from other sources that are known to make growth factors that might be stimulatory to CWR22. At the beginning of the third year of this project, experiments were in progress with the coinjected line NCI-H23 obtained from the Biological Testing Branch of the National Cancer Institute. The coinjected irradiated NCI-H23 did not cause acceleration of the growth of CWR22. SF-295 is a line that, as described previously, had shown quite variable results in different previous experiments. Because some of these experiments were encouraging, SF-295 was tested again. In this, the final experiment with SF-295, it was ineffective in stimulating the growth of CWR22. The xenograft line, K-562, was also tested in graded doses as a lethally irradiated "feeder" cell for CWR22. It did not stimulate increased growth of CWR22.

On learning of an article published in Cancer Research in 2001 (Gho et al., 2001), we decided to test, as an irradiated feeder cell, PC3 prostate cancer cells transfected by intercellular adhesion molecule-1 (ICAM-1) and anti-sense ICAM-1. Gho et al. had found that ICAM-1 stimulated the growth of PC3 cells in chick chorioallantoic membranes and that ICAM-transfected PC3 cells grew more rapidly than anti-sense transfected PC3 cells both in that system and as xenografts in nude mice. Their laboratory generously supplied us with the cells. Unfortunately, we found (a) very little difference in the rates of growth of these two transfectants in nude mice and (b) no ability of either transfectant to accelerate the growth of CWR22 as lethally irradiated feeder cells.

The number of lines that we tested in the third year of this project was smaller than anticipated because of changes in our technical help. Because of an eleven-hour craniotomy for the subtotal resection of a very extensive meningioma followed by gamma-knife irradiation of the remaining tumor, the principal investigator's activity was slowed down. His (my, Thomas G. Pretlow) surgery left him with diplopia and other eye problems. After a decade working with xenografts in the Pretlow laboratory, his most senior technician, Joe Giaconia, in the xenograft area left Ohio to take a job with a large drug manufacturer in 2001. He told one of his colleagues that he left because he thought that Dr. Pretlow would retire soon in light of his disability. In 2002, the principal investigator arrived at the same conclusion and informed the laboratory that after his NIH and DOD grants finished, he planned (a) not to apply for additional grants and (b) to close his laboratory. This resulted in another senior technician's decision to take employment elsewhere. The resultant changes in technical help have resulted in time spent training new research assistants and slowed progress. We retained unspent funds with the goal of completing the testing of coinjected, lethally irradiated cell lines with funds carried over to an additional year.

Specific aim 3 (also task 3) is dependent upon success in specific aim 2. While many experiments related to specific aim 2 have been carried out as described in this and previous reports, we have not succeeded in finding the needed line of cells to act as irradiated feeder cells for CWR22. Experiments continue.

Specific aim 4 (also task 4) was being pursued with the collection of the clinical data described in that aim for use in future correlations between clinical data and xenograft behavior. As described under "Rationale" for specific aim 4 in the original proposal, "It is pos-

sible that clinical and pathological features of the PCA patients whose PF cells grow as xenografts are correlated with the biology of the xenografts. The ability of tumor to grow to form xenografts may predict aggressiveness in the patient....We shall begin to look for correlations among these data and the behavior of the xenografts, i.e., growth, rate of growth, karyotype, patterns of metastasis, etc." Since no xenografts were obtained, these studies are not fruitful.

Key Research Accomplishments

Prostatic fluid cells from eighteen patients have been injected into nude mice. While the cells from the fluids of many patients caused variable, low-level elevations of PSA in the blood of nude mice for several months, no patient's cells formed tumors. We had previously demonstrated that prostate cancer cells from primary human prostate cancers could survive with almost no significant growth in nude mice. The assay that we developed and employed, the coinjection of small doses of xenograft cells with irradiated "feeder" cells is imprecise and probably not very useful in its current form.

Reportable Outcomes

There are no reportable outcomes.

Conclusions

The only two conclusions that seem important are that (a) prostate cancer cells from prostatic fluids of a significant proportion of patients with primary prostate cancers can survive for a few months in nude mice and (b) they do not grow rapidly enough to form transplantable xenografts with the technology that is currently available.

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Appendices: No appendices are included.